A stereospecific analysis of triglycerides

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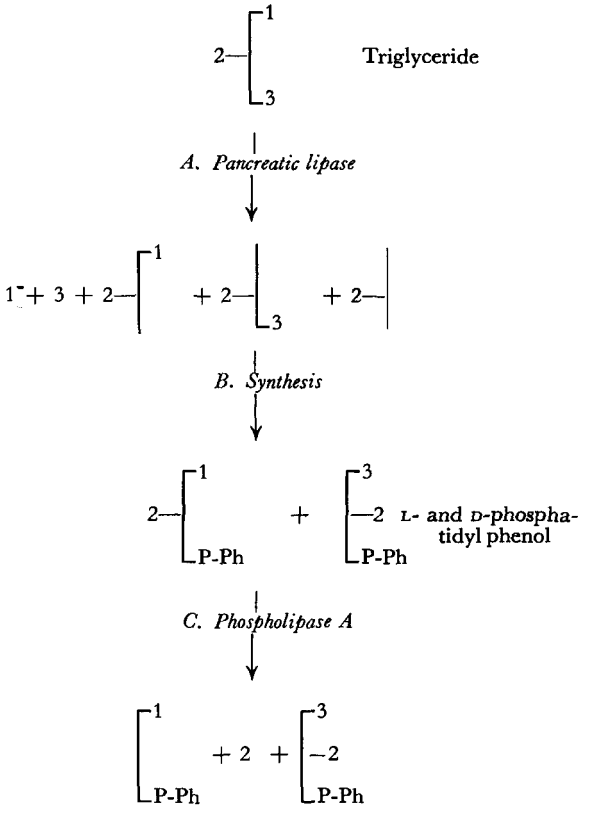
SUMMARY A method is presented for the analysis of the fatty acid compositions in each of the three positions of a triglyceride. Diglycerides are obtained from the triglyceride by lipolysis with pancreatic lipase, and converted to a mixture of D- and L-phosphatidyl phenol. The L-phosphatide is then hydrolyzed by phospholipase A, leaving a lysophosphatide with the fatty acid in position 1, free fatty acid from position 2, and unhydrolyzed D-phosphatidyl phenol. The fatty acid composition in position 3 of the original triglyceride is obtained by calculation.

The method was tested on two triglyceride mixtures with known fatty acid distributions. A natural triglyceride, corn oil, was analyzed. The distribution of the major fatty acids between positions 1 and 3 was found to be nearly random.

KEY WORDS stereospecific analysis · triglyceride analysis · triglyceride structure · corn oil · lipolysis · phospholipase A · phosphatidyl phenol · lysophosphatide phenol · fatty acid distribution.

THE DISTRIBUTION of fatty acids between the α- and β-positions of a triglyceride can be determined by using pancreatic lipase (EC 3.1.1.3). This enzyme hydrolyzes the ester bonds in the α-, or 1- and 3-, positions (1, 2). Its action, however, is not stereospecific in that it makes no distinction between the positions 1 and 3 of a 1,2,3-triacyl L-glycerol[1] (3, 4). The analytic method reported here utilizes both pancreatic lipase and a stereospecific lipolytic enzyme, phospholipase A (EC 3.1.1.4), to determine the fatty acid composition in each of the three positions. In the above scheme the skeletal backbones represent glycerol; P-Ph is phosphoryl phenol; and 1, 2, and 3 stand for the fatty acids in these positions *in the triglyceride*.[2]



The first step in the method is a partial hydrolysis of the triglyceride with pancreatic lipase (reaction *A*). From

[1] In this paper, tri- and diglycerides are described as derivatives of L-glycerol; the hydroxyl group at 2 is written to the left in the Fischer projection formulae. If the prefix L is omitted the nomenclature becomes identical with that proposed by H. Hirschmann (*J. Biol. Chem.* **235**: 2762, 1960). The DL system is preferred here because it allows the convenient description of the isomeric phosphatides as L- and D-phosphatidyl derivatives.

[2] And in the L-phosphatides; in D-phosphatidyl phenol the carbinol group carrying fatty acid 3 would be given the number 1 by virtue of the DL convention.

among the reaction products the diglyceride fraction, consisting of 1,2-diacyl and 2,3-diacyl L-glycerol[3], is isolated. These diglycerides are converted (reaction *B*) into a mixture of L- and D-phosphatides (in this study, into phosphatidyl phenol). Phospholipase A from snake venom (reaction *C*) will attack only the L-phosphatide (5, 6), liberating the fatty acids from the 2-position (6–8) and leaving a lysophosphatide with the fatty acid from position 1, and a D-phosphatide with fatty acids 2 and 3. Fatty acids 1 and 2 can be analyzed directly; fatty acid 3 is found by difference.

The method demands that no isomerizations occur in any of the reactions, and that there is no discrimination, in the form of a greatly different reaction rate, against any of the common fatty acids. Analysis of two triglycerides with known fatty acid distributions showed that these conditions are reasonably well fulfilled. The method was then applied to one natural triglyceride, corn oil.

MATERIALS AND METHODS

Triglycerides

The fatty acid composition in positions 1 and 2 of an egg lecithin was determined (8). Reaction of the lecithin with phospholipase D (EC 3.1.4.3) (8) yielded a diglyceride (1,2-diacyl L-glycerol) mixture which was then acylated in position 3 with myristoyl chloride in pyridine–chloroform. The theoretical structure of the resulting Triglyceride A is given in Table 1 under Synthesis.

Preparation of the second triglyceride began with the acylation of 1,2-acetonyl L-glycerol (9) with an equimolar mixture of myristoyl chloride and stearoyl chloride in pyridine–chloroform. The acetonyl group was then removed with boric acid (10), and the resulting 3-acyl L-glycerol was analyzed for its fatty acid composition and then acylated with the theoretical amount of fatty acid chlorides prepared from linseed oil. The triglyceride was purified by passage through basic aluminum oxide in chloroform (to remove any free fatty acids) followed by chromatography on silicic acid (11) to remove any partial glycerides. The theoretical structure of this preparation (Triglyceride B) is given in Table 2 under Synthesis.

The corn oil used was a commercial product, Mazola corn oil, which had been purified by chromatography on aluminum oxide and silicic acid.

Analysis of Fatty Acids

Fatty acids were analyzed as their methyl esters with an Aerograph Hi-Fy gas chromatograph (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.). The methyl esters were prepared by esterification of the fatty acids with methanol–boron trifluoride (12) or by methanolysis of the lipids by boiling with the same reagent for 10 min, with addition of chloroform to dissolve the triglycerides. Methanolyses carried out for 5 and for 20 min yielded methyl esters of identical composition. For gas–liquid chromatography (GLC), an aluminum column, 8 ft in length and $^1/_8$ inch o.d., was employed with a stationary phase of ethylene glycol succinate–silicone polyester (EGSS-X), 10% on Gas Chrom P, 100–120 mesh (Applied Science Laboratories, State College, Pa.). The carrier gas was nitrogen at 45 psi, and the column temperature 190°.

Quantification was based on the assumption that the response of the hydrogen flame detector was proportional to the concentration of carbon atoms carrying hydrogen atoms (13). The relative response areas, obtained by multiplying peak heights by retention times (14), were consequently divided by the fatty acid chain length to yield concentrations in moles per cent. Quantitative results with National Heart Institute Fatty Acid Standards (mixtures A and D) (15) agreed with the stated composition data with a relative error of less than 2% for major components (>10% of total mixture) and <10% for the minor component (palmitoleic acid, 7.2 moles % of total mixture).

Methanolysis of the phosphatides yielded some phenol, which accompanied the methyl esters. In the gas chromatograms the phenol can be recognized by its strong tailing effect. On the EGSS-X column it follows closely the myristic acid methyl ester, for which it might be mistaken, but the separation is quite clear; the esters 15:0, 14:1, and 14:2, however, would be covered by the phenol peak. In the analysis of lipids containing these acids, the phenol should, therefore, be removed. This might be done by repeated extraction of the solution of methyl esters with aqueous Na_2CO_3 or by a chromatographic method.

Thin-Layer Chromatography

Silicic acid plates (10 × 20 cm and 0.15 cm thick) were prepared according to Vogel et al. (16). With the solvent mixture chloroform–methanol–3% aqueous ammonia 63:30:7 (v/v/v), the products of reaction *C* had the following approximate R_F values: phosphatidyl phenol, 0.9; fatty acids, 0.75; lysophosphatidyl phenol, 0.6. Increasing the proportion of chloroform led to lower R_F values but did not further improve the separation. An equally effective solvent mixture was ether–methanol–water–triethylamine 85:13:1:1, which gave R_F values of 0.6, 0.45, and 0.25 for the three compounds. The separations appeared to be sharper if the plates were kept over concentrated aqueous ammonia for 20 min prior to application of the samples. About 10 mg of

[3] D-Diglyceride and L-diglyceride in the Fischer nomenclature, in which the oxidizable free carbinol group is always given the number 1.

TABLE 1 FATTY ACID DISTRIBUTION IN TRIGLYCERIDE A AS PREDICTED ACCORDING TO PARTIAL SYNTHESIS, AND FOUND ON ANALYSIS

	Position of Triglyceride	Fatty Acid				
		14:0	16:0	18:0	18:1	18:2
			*moles %**			
Synthesis	1		67	24	6	
	2		5		63	29
	3	100				
Analysis	1	1	64	25	5	
	2	2	3		60	33
	3	97	4		(−2)†	

* Fatty acids of less than 1 mole % are omitted.
† See Discussion.

lipid was fractionated on each plate. Spots were localized with a bromothymol blue spray (40 mg in 100 ml of 0.01 N aqueous NaOH plus 5 ml of concentrated ammonia). They were then scraped off, and eluted with chloroform–methanol–water 50:45:5.

Calculations

The composition of fatty acid 1 is obtained by analysis of the lysophosphatide formed in reaction *C*. Fatty acid 2 is released by phospholipase A (reaction *C*); it is also found as monoglyceride after reaction *A*. Fatty acid 3 can be determined only indirectly, by subtraction of 1 from 1 + 3, or of 1 and 2 from the total triglyceride, or of 2 from 2 + 3 (the unattacked D-phosphatide after reaction *C*). The third calculation requires that reaction *C* has gone to completion. In subtracting, it must be remembered that the fatty acid compositions, as listed in the tables, always add up to 100 moles % or 1 mole, whereas they may represent 1 or 2 moles of fatty acids. For instance, the D-phosphatidyl phenol (Table 3) contains 2 moles of fatty acids, 2 and 3; therefore, since myristic occurs in 24.4 moles % in this phosphatide, and in 1.7% in position 2 (Table 2), its concentration is (24.4 × 2) − 1.7 or 47.1 moles % in position 3 (Table 2).

PROCEDURES AND RESULTS

Triglyceride A

This triglyceride (2 g) was subjected to the action of pancreatic lipase (Steapsin, Sigma Chemical Co., St. Louis, Mo.) according to Mattson and Volpenhein (17). The reaction was stopped after approximately one half of the triglycerides had been hydrolyzed, and the diglyceride fraction was obtained by chromatography on silicic acid (11). The diglycerides, 0.42 g (about 0.7 mmole), dissolved in 2 ml of ether, were added with cooling to 0.23 ml (1.5 mmoles) of freshly distilled phenyl dichlorophosphate (K & K Laboratories, Jamaica, N.Y.) dissolved in a mixture of 2 ml of chloroform and 2 ml of pyridine. After 14 hr at room temperature, 6 ml of pyridine and 1 ml of water were added with cooling. One hour later 30 ml each of chloroform and methanol and 25 of water were added, the mixture was shaken and the chloroform layer recovered. One milliliter of triethylamine was added, the solvent evaporated, and the triethylammonium salt of the phosphatidyl phenol (0.59 g) dissolved in 3 ml of ether. Thirty milliliters of 0.05 M aqueous triethylammonium bicarbonate, pH 7.5 (prepared by saturating triethylamine in water with carbon dioxide), 0.1 ml of 0.1 M $CaCl_2$, and 10 mg of phospholipase A (snake venom, *Crotalus atrox*) were added. The mixture was shaken vigorously for 4 hr at room temperature under an atmosphere of nitrogen, and then taken to dryness on a rotary evaporator. The products were separated on silicic acid plates with the

TABLE 2 FATTY ACID DISTRIBUTION IN TRIGLYCERIDE B AS PREDICTED ACCORDING TO SYNTHESIS, AND FOUND ON ANALYSIS

	Position of Triglyceride	Fatty Acid						
		14:0	16:0	16:1	18:0	18:1	18:2	18:3
					moles %			
Synthesis	1		7.4	0.3	3.5	15.8	16.8	56.3
	2		7.4	0.3	3.5	15.8	16.8	56.3
	3	49.8			50.2			
Analysis	1	1.7	7.9	0.4	4.3	14.9	17.0	53.8
	2*	1.1	8.0	0.3	3.3	15.4	16.4	55.4
	2†	1.7	7.9	0.5	3.8	15.7	16.0	54.4
	3‡	47.0	(−1.1)‖	(−0.2)‖	49.6	1.3	0.2	3.4
	3§	47.1	1.1	0.2	47.0	1.5	1.4	1.8

* Monoglyceride. This analysis and the following are described under Procedures and Results, Triglyceride B, fourth paragraph.
† Free fatty acids from L-phosphatide.
‡ Triglyceride minus 1 minus 2*.
§ D-Phosphatide (Table 3) minus 2†.
ee Discussion.

solvent containing chloroform. The fatty acids of the lysophosphatide (position 1) were analyzed, as their methyl esters, by GLC. The monoglycerides (position 2) and the fatty acids (positions 1 and 3) isolated after lipolysis of the synthetic triglyceride were also analyzed and the fatty acid composition of 3 was calculated as the difference of 1 + 3 (the free fatty acids from lipolysis) and 1 (the lysophosphatide) (Table 1).

Triglyceride B

The lipolytic method of Mattson and Volpenhein (17) was modified here to minimize the possibility of acyl migration on the diglyceride. The reaction time in the synthesis of the D,L-phosphatide was much reduced, and the phosphatide was purified by chromatography on silicic acid.

The triglyceride, 2 g, was suspended in 36 ml of M tris buffer of pH 7.5; then 0.4 ml of 45% $CaCl_2$, 0.16 ml of a 1% solution of Difco bile salt No. 3, a few drops of bromophenol blue solution, and 200 mg of pancreatin in 2 ml of buffer were added, and the mixture was shaken by hand for 6 min at room temperature. This procedure, as all following ones, was carried out under nitrogen. It had been established by previous experiments with olive oil that the conditions would result in a good yield of diglycerides. The reaction was stopped by the addition of 20 ml of ethanol and 20 ml of ether, and 6 N HCl was then added until the indicator changed to a blue-green color (pH 4). The lipids recovered from three ether extractions were separated on silicic acid (11), the diglycerides (516 mg) and the monoglycerides (269 mg) were collected and their fatty acid compositions determined.

The diglycerides, 258 mg (about 0.4 mmole) in 2.5 ml of ether, were added, over several minutes, to a solution of 0.23 ml (1.5 mmoles) freshly distilled phenyl dichlorophosphate in 2 ml of chloroform (distilled from P_2O_5) and 2 ml of pyridine (distilled from BaO). The reaction flask was cooled by ice water. After 2 hr at room temperature, 5 ml of pyridine and 1 ml of water were added, again with cooling. After one more hour at room temperature, 30 ml each of chloroform and methanol and 25 ml of water were added, the mixture was shaken, and the chloroform layer recovered. One milliliter of concd aqueous ammonia was added, the solvent evaporated, and the residue taken up in a few milliliters of ether. It was placed on a silicic acid column (Mallinckrodt, 16 g, 9 × 2 cm), and eluted with one column volume (32 ml) of ether. The eluted material (17 mg) consisted of diglycerides, fatty acids, and monoglycerides. The second solvent, ether–methanol–concd aqueous ammonia 88:10:2, six column volumes (190 ml), eluted D,L-phosphatidyl phenol, which was isolated as the triethylammonium salt by evaporating the solvent after addition of 1 ml of triethylamine. The material thus obtained (346 mg, 96% of theory) gave one spot on thin-layer chromatography. It contained 3.54% phosphorus (calculated for the dilinolenate, mol wt 870.2: P, 3.57%). The structure of the compound follows from the synthesis.[4]

TABLE 3 FATTY ACID COMPOSITIONS OF INTERMEDIATES IN THE ANALYSIS OF TRIGLYCERIDE B

	Fatty Acids						
	14:0	16:0	16:1	18:0	18:1	18:2	18:3
	moles %						
D,L-Diglyceride, theory	12.5	5.4	0.2	15.2	11.9	12.5	42.4
D,L-Diglyceride, found	11.4	4.6	0.4	16.2	12.3	13.1	42.0
D,L-Phosphatidyl phenol	11.7	5.0	0.3	16.0	12.5	12.9	41.6
D-Phosphatidyl phenol	24.4	4.5	0.3	25.4	8.6	8.7	28.1

The D,L-phosphatidyl phenol (250 mg) was treated with snake venom as described for Triglyceride A. The products were separated on silicic acid plates with the solvent containing ether and analyzed for their fatty acid composition. In Table 2, the first line under Analysis, position 1, gives the fatty acid composition of the lysophosphatidyl phenol. Line 2* is the monoglyceride isolated after lipolysis of the synthetic triglyceride, and line 2† is the mixture of fatty acids released from the phosphatidyl phenol by phospholipase A. Line 3‡ gives the composition of position 3 as derived from the composition of the triglyceride minus 2* minus 1. The analysis of the D-phosphatidyl phenol (Table 3) minus 2† (Table 2) yields 3§ (Table 2), the second independent determination of the fatty acid composition in position 3.

The fact that it was possible to calculate the approximate theoretical composition of fatty acid 3 (Table 2, last line) from the recovered D-phosphatidyl phenol indicates that the enzymatic hydrolysis of the L-compound had gone to completion. For additional proof, a separation of the hydrolysis products on a silicic acid column was attempted. An amount corresponding to 100 μmoles of the original D,L-phosphatidyl phenol was placed on a column (16 g of Mallinckrodt silicic acid, 9 × 2 cm). Elution with ether (32 ml, one column volume) yielded a mixture of fatty acids and phosphatidyl phenol which could not be satisfactorily separated by rechromatography. The next solvent, ether–methanol–concd aqueous ammonia 88:10:2 (190 ml, six column volumes) eluted more phosphatidyl phenol. The lysophosphatide

[4] A possible by-product, the phosphoric acid triester bis-phosphatidyl phenol, would not be attacked by phospholipase A (6). Since the following results show that one-half of the DL-phosphatide is hydrolyzed by this enzyme, the triester can be present only in trace amounts.

TABLE 4 FATTY ACID DISTRIBUTION IN CORN OIL

Position	Fatty Acids, *Moles %*					
	16:0	16:1	18:0	18:1	18:2	18:3
1	24.7	0.8	4.7	23.0	45.6	1.1
2*	1.7	0.2	0.2	26.4	70.2	1.1
2†	2.0	0.4	0.5	26.9	69.0	1.0
3‡	17.5	0.4	3.3	31.4	45.8	1.4
3§	20.3	0.5	4.9	25.0	45.8	2.7

* Monoglyceride.
† Free fatty acids from L-phosphatide.
‡ Triglyceride minus 1 minus 2*.
§ D-phosphatide (not listed) minus 2†.

was then recovered with two column volumes (64 ml) of ether–methanol–concd aqueous ammonia 70:28:2. The material, as the triethylammonium salt, contained 5.02% phosphorus (calculated for the linolenate, mol wt 609.8: P, 5.10%). Its fatty acid composition was identical with that of the lysophosphatidyl phenol obtained by thin-layer chromatography. The yield was 32 mg or 53 μmoles. It appears, therefore, that the enzymatic hydrolysis of the L-phosphatide was complete.

Corn Oil

The purified corn oil was analyzed as outlined under Triglyceride B. Table 4 shows the results.

DISCUSSION

The most obvious application of the method would be the analysis of natural triglycerides; for this reason, the two synthetic preparations were chosen to resemble common natural fats in their fatty acid composition, and perhaps in their structure. The method should also prove useful in metabolic studies. Clearly, in addition to the fatty acid pattern, the distribution of radioactivity can also be analyzed.

The tables illustrate the accuracy of the method. For the major fatty acids the analyses are satisfactory, while the results for the trace components are clearly less reliable. There are several reasons for this lack of accuracy. First, there are the possible errors of gas-chromatographic determinations (15). Second, the stereospecific analysis cannot be expected to exceed the accuracy of the lipolytic method which is part of it (reaction *A*). A major problem here may be that of discrimination between different fatty acids. It is known, for instance, that the short-chain fatty acids of milk fat are split off faster than the long-chain acids (18), and a comparison of the diglycerides obtained from Triglyceride B with the diglyceride expected (Table 3) indicates a slightly preferential hydrolysis of myristic acid esters. These would be the main sources of error in the analysis of positions 1 and 2. The good agreement (except for the trace components) of the two analyses of position 2 should be noted. These data prove that there have been no changes in composition or structure of the lipids during the entire procedure.

A more serious problem arises in the analysis of 3. While 1 and 2 can be analyzed directly, 2 even in two independent ways, 3 can be determined only by indirect methods. Fortunately, two partly independent analyses are available, which can, or rather should, be compared in all cases, but there remain large relative errors for minor or trace components: acids appear in position 3 which should not be present at all, and even negative concentrations may result from the calculations. These errors stem from the cumulative effect of inaccuracies of GLC and lipolysis. There is, of course, the possibility that some isomerization took place during the preparation of the synthetic triglycerides, although isomerization would not result in the negative concentration values. It may account, however, for the occurrence of myristic acid in the direct analyses of positions 1 and 2 (Tables 1 and 2).

Taking into consideration these limitations we may interpret the analysis of corn oil (Table 4) as follows: the distribution of the major fatty acids between positions 1 and 3 is almost random; there is only a slight excess of palmitic acid in 1 and of oleic acid in 3 position. Of the minor fatty acids, stearic acid is evenly distributed between 1 and 3. Linolenic acid is present in both 1 and 3 positions; so is the trace component, palmitoleic acid.

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REFERENCES

1. Savary, P., and P. Desnuelle. *Biochim. Biophys. Acta* **21:** 349, 1956.
2. Mattson, F. H., and L. W. Beck. *J. Biol. Chem.* **219:** 735, 1956.
3. Tattrie, N. H., R. A. Bailey, and M. Kates. *Arch. Biochem. Biophys.* **78:** 319, 1958.
4. Karnovsky, M. L., and D. Wolfe. *4th Intern. Congr. Biochem.*, Abstr. Communs., 208, 1958.
5. Long, C., and I. Penny. *Biochem. J.* **65:** 382, 1957.
6. Van Deenen, L. L. M., and G. H. De Haas. *Biochim. Biophys. Acta* **70:** 538, 1963.
7. Tattrie, N. H. *J. Lipid Res.* **1:** 60, 1959.
8. Hanahan, D. J., H. Brockerhoff, and E. J. Barron. *J. Biol. Chem.* **235:** 1917, 1960.
9. Baer, E. In *Biochemical Preparations*, edited by E. G. Ball. John Wiley and Sons, Inc., New York, 1952, Vol. 2, p. 31.
10. Martin, J. B. *J. Am. Chem. Soc.* **75:** 5482, 1953.
11. Barron, E. J., and D. J. Hanahan. *J. Biol. Chem.* **231:** 493, 1958.
12. Metcalfe, L. D., and A. A. Schmitz. *Anal. Chem.* **33:** 363, 1961.

13. Ettre, L. S., and F. J. Kabot. *J. Chromatog.* **11:** 114, 1963.
14. Tandy, R. K., F. T. Lindgren, W. H. Martin, and R. D. Wills. *Anal. Chem.* **33:** 665, 1961.
15. Horning, E. C., E. H. Ahrens, Jr., S. R. Lipsky, F. H. Mattson, J. F. Mead, D. A. Turner, and W. H. Goldwater. *J. Lipid Res.* **5:** 20, 1964.
16. Vogel, W. C., W. M. Doizaki, and L. Zieve. *J. Lipid Res.* **3:** 138, 1962.
17. Mattson, F. H., and R. A. Volpenhein. *J. Lipid Res.* **2:** 58, 1961.
18. Clement, G., J. Clement, and J. Bezard. *Biochem. Biophys. Res. Comm.* **8:** 238. 1962.